

Ecotoxicology (2012) 21:439–455  
DOI 10.1007/s10646-011-0804-0

# Transgenic nematodes as biosensors for metal stress in soil pore water samples

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Accepted: 24 September 2011 / Published online: 30 October 2011  
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**Abstract** *Caenorhabditis elegans* strains carrying stress-reporter green fluorescent protein transgenes were used to explore patterns of response to metals. Multiple stress pathways were induced at high doses by most metals tested, including members of the heat shock, oxidative stress, metallothionein (*mtl*) and xenobiotic response gene families. A mathematical model (to be published separately) of the gene regulatory circuit controlling *mtl* production predicted that chemically similar divalent metals (classic inducers) should show additive effects on *mtl* gene induction, whereas chemically dissimilar metals should show

interference. These predictions were verified experimentally; thus cadmium and mercury showed additive effects, whereas ferric iron (a weak inducer) significantly reduced the effect of mercury. We applied a similar battery of tests to diluted samples of soil pore water extracted centrifugally after mixing 20% w/w ultrapure water with air-dried soil from an abandoned lead/zinc mine in the Murcia region of Spain. In addition, metal contents of both soil and soil pore water were determined by ICP-MS, and simplified mixtures of soluble metal salts were tested at equivalent final concentrations. The effects of extracted soil pore water (after tenfold dilution) were closely mimicked by mixtures of its principal component ions, and even by the single most prevalent contaminant (zinc) alone, though other metals modulated its effects both positively and negatively. In general, mixtures containing similar (divalent) metal ions exhibited mainly additive effects, whereas admixture of dissimilar (e.g. trivalent) ions often resulted in interference, reducing overall levels of stress-gene induction. These findings were also consistent with model predictions.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10646-011-0804-0) contains supplementary material, which is available to authorized users.

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**Keywords** Transgenic *C. elegans* · GFP reporters ·  
Stress-response pathways · Metal contamination ·  
Soil water · Mathematical modelling

## Introduction

The free-living soil nematode, *Caenorhabditis elegans* (Rhabditidae, Nematoda), has become a favoured model organism in many areas of biology, thanks to its complete somatic cell lineage (Sulston et al. 1983), its fully sequenced genome (*C. elegans* Sequencing Consortium 1998), and a variety of genetic tools including transgenic strains generated by micro-injection (Mello et al. 1992) and genome-wide

RNA interference by feeding (Kamath et al. 2003). As a laboratory model, *C. elegans* offers many experimental advantages, including its small size (~1 mm as adults), transparency, rapid life cycle (3.5 days from zygote to egg-laying adult at 25°C), large brood size (c. 250 self-fertilised progeny per hermaphrodite adult), and simple culture on lawns of *E. coli* in agar-containing Petri dishes (Brenner 1974; Riddle et al. 1997). These advantages have been extensively exploited in ecotoxicology over the past 20 years, initially for aquatic testing of metal toxicity (Williams and Dusenbery 1990). In the wild, *C. elegans* feeds mainly on bacteria in decaying matter such as compost (Kenney et al. 2006; Chen et al. 2006), and inhabits the film of water around soil particles; it is therefore suitable for toxicity testing in both aquatic and soil environments (reviewed by Leung et al. 2008). However, counterbalancing these many advantages, *C. elegans* is also much less sensitive to the majority of environmental stressors as compared with other test species such as *Daphnia*. This probably reflects the formidable array of cellular defence pathways which *C. elegans* may need to deploy occasionally as a consequence of its opportunistic lifestyle in the soil.

Numerous studies have established the utility of transgenic *C. elegans* strains as biosensors of metal pollution, using well-characterised heat-shock (e.g. *hsp-16.1* and *hsp-16.2*; Stringham and Candido 1994; Link et al. 1999; David et al. 2003) and metallothionein (notably *mtl-2*; Freedman et al. 1993; Cioci et al. 2000; Swain et al. 2004; Ma et al. 2009a) output genes as suitable markers likely to be induced by metal stress. Less thoroughly investigated are markers of oxidative stress, such as catalase and superoxide dismutase genes; these may be up-regulated either directly by redox-active metals, or may respond indirectly, e.g. through cadmium (non redox-active) binding to reduced glutathione. In addition, genome-wide gene-array studies have shown unexpectedly strong metal induction of classic xenobiotic response genes (e.g. Cui et al. 2007), such as those encoding certain phase I cytochrome P450s (*cyp* genes) or phase II glutathione-S-transferases (*gst* genes). In part, the activation of multiple stress pathways by metals may reflect co-ordinate transcriptional control by master regulators such as the FOXO transcription factor (TF) DAF-16, whose known targets include *cyp-34A9* as well as *hsp-16.1/16.2*, *sod-3* and *mtl-1* (Murphy et al. 2003). DAF-16 is down-regulated by food signals acting through insulin-like ligands and the DAF-2 receptor. When food is limiting, insulin signalling is reduced and DAF-16 activated, resulting in the up-regulation of many stress-response genes (above), which act to defend cells against damage and so prolong lifespan (reviewed by Kenyon 2010). Another master regulator controlling multiple stress-response pathways is the zinc-finger transcription factor SLR-2 (Kirienko and Fay 2010). Testing multiple output genes simultaneously allows insight into the patterns

of stress response within the test organism, and this approach is greatly facilitated by the use of green fluorescent protein (GFP) reporters (Chalfie et al. 1994; de Pomerai et al. 2008). Although hitherto applied only to single output genes, similar tests can also be used to assess the toxicity of field samples of metal-contaminated water and soils (Mutwakil et al. 1997; Power and de Pomerai 1999), and have previously demonstrated synergistic effects for metal mixtures (Power et al. 1998; Chu and Chow 2002).

There are now several *C. elegans*-based standard protocols for assessing soil toxicity using whole-organism endpoints such as lethality (Donkin and Dusenbery 1993; Freeman et al. 1999) or impairment of growth and brood size in sediment samples (Traunspurger et al. 1997). With suitable modifications such as centrifugal extraction of soil pore water (Power and de Pomerai 1999), other endpoints including feeding inhibition (Jones and Candido 1999) can also be tested. These provide an overview of how soil-dwelling biota (including free-living nematodes such as *C. elegans*) respond to the prevailing soil conditions—for instance metal and/or pesticide contamination. Whereas extracted soil pore water is convenient for determining short-term acute toxicity, longer-term studies of chronic toxicity require the test worms to be cultured in the soil environment and later recovered, e.g. by flotation on colloidal silica (Donkin and Dusenbery 1993). This raises problems both of recovery and of contamination with endogenous nematode populations when studying natural soils. The latter can be circumvented by using transgenic GFP-expressing strains (since only test worms can express GFP), but it remains difficult to standardise worm recovery protocols for use in rapid-throughput screening assays requiring equal worm numbers. For this reason, we have only tested soil pore water (where the number of worms per assay can be readily controlled) within this study, though we are well aware that this will underestimate the toxic effects of less soluble metals (e.g. lead) present in soils, and that a whole-soil assay might be preferable. Rather than focussing on just one or a few such transgenic strains, we have chosen a battery of GFP-reporter transcriptional fusions (Hunt-Newbury et al. 2007) that monitor the expression of multiple stress-inducible output genes acting in different defensive or cell-repair pathways. Our 24 test genes include 7 members of the heat-shock pathway, 3 metal-response genes, 7 oxidative stress genes, 5 genes involved in xenobiotic metabolism, plus the p53 orthologue *cep-1* and the master transcriptional regulator *daf-16* (details in “Materials and Methods” section).

This paper presents the principal results from three linked studies. We first present an overview of the response patterns of all 24 strains to zinc and 10 other metals; full details (for all doses and time points) are available as Supplementary Material. One classic protective response to metal stress involves the up-regulation of genes encoding *mtls*, which are

small cysteine-rich proteins that can sequester excess metals and assist in their excretion. In *C. elegans* there are only two *mtl* genes (*mtl-1* and *mtl-2*; Freedman et al. 1993; Cioci et al. 2000; Swain et al. 2004), whose induction probably involves a feedback loop comprising the GATA TF ELT-2 plus an unknown metal-sensitive repressor (Moilanen et al. 1999). In our second study, we have modelled this regulatory circuit mathematically (Haque 2011); for simple two-component metal mixtures, the *mtl* model predicts additive effects for mixtures of chemically similar divalent ions (classic inducers), but interfering effects for mixtures of dissimilar ions. Both of these predictions have been verified experimentally using an *mtl-2::GFP* reporter strain. In our third study, we report how selected strains respond to diluted samples of soil pore water derived from the site of a disused lead/zinc mine in the Murcia region of south-eastern Spain. This study site has been an important mining area for over 2,500 years, with rich deposits of lead, zinc and iron ores. Although mining activity ceased in 1991, high concentrations of these metals in the soil are attributable to intense mining and smelting activities in the past. The mine tailings also contain high concentrations of other heavy metals, especially mercury, cadmium, copper and arsenic. The effects of soil pore water from this site are matched by artificial mixtures of the component metal ions at the same concentrations (and even by the main contaminant, zinc, on its own). There are interesting variations in response to the different metal mixtures tested, which seem to imply that the broad predictions of our *mtl* model may also hold true for metals in the more complex mixtures tested here, at least for the *mtl-2* gene. Moreover, similar patterns of response are evident for a small heat-shock gene (*hsp-16.2*) and a highly inducible xenobiotic response gene (*cyp-35A2*; Menzel et al. 2001), though not for an oxidative stress gene (*sod-4*, encoding an extracellular superoxide dismutase). Relationships between these response patterns and the inferred modes of regulation of these stress genes will be explored briefly in the “Discussion” section. Overall, we have demonstrated the utility of multiple *C. elegans* strains carrying inducible GFP-reporter transgenes for exploring complex patterns of stress responses to metals, both singly and in mixtures. Surprisingly, interference effects are apparent as well as the expected additive effects in mixtures, although this may apply only to individual gene expression responses and not necessarily to the overall toxicity.

## Materials and methods

### Materials

Strain sources were as follows: PC161 (*hsp-16.1::GFP::lacZ*) was developed in-house (David et al. 2003); thanks

are due to Joel Rothman for JR2474 (*cep-1::GFP*; Derry et al. 2001), Chris Link for CL2050 (*hsp-16.2::GFP*; Link et al. 1999), Cynthia Kenyon for CF1553 (*sod-3::GFP*), Ralph Menzel for a strain carrying pPD97 87-35A2prIII-GFP (*cyp-35A2::GFP*; Menzel et al. 2001) and the *Caenorhabditis* Genetics Center (University of Minnesota, funded by NIH National Center for Research Resources) for TJ356 (*daf-16::GFP*). Other strains were supplied as integrated promoter::GFP fusions by the Baillie Genome GFP Project (Simon Fraser University, Burnaby, Vancouver, Canada; see Hunt-Newbury et al. 2007), each containing ~3 kb of upstream promoter sequence (apart from BC20306 which contains only 250 bp of promoter separating the *cyp-34A9* gene from its upstream neighbour *cyp-34A10*). These are: BC17553 (T09A12.2 glutathione peroxidase designated gpA::GFP), BC20303 (*hsp-6::GFP*), BC20305 (C11E4.1 glutathione peroxidase designated gpB::GFP), BC20306 (*cyp-34A9::GFP*), BC20308 (*hsp-3::GFP*), BC20309 (*mtl-1::GFP*), BC20314 (*elt-2::GFP*), BC20316 (*gst-1::GFP*), BC20329 (*skn-1::GFP*), BC20330 (*gst-4::GFP*), BC20333 (*sod-4::GFP*), BC20334 (*cyp-29A2::GFP*), BC20336 (*ctl-2::GFP*), BC20337 (*hsf-1::GFP*), BC20342 (*mtl-2::GFP*), BC20343 (*hsp-60::GFP*), BC20349 (C12C8.1, now re-designated *hsp-70::GFP*) and BC20350 (*sod-1::GFP*). All of the fusion gene arrays were integrated by X irradiation, and stable transgenic lines were outcrossed (4×). Inducibility and expression patterns for all 24 genes are available at [www.wormbase.org](http://www.wormbase.org). Strain PD4251 was used as a control strain that should be unresponsive to chemical stress, and was obtained from the *Caenorhabditis* Genetics Center (details above). PD4251 contains 3 transgenes: pSAK2 comprising a *myo-3* muscle myosin promoter driving a nuclear-targeted *lacZ* reporter; pSAK4 comprising a *myo-3* promoter driving a mitochondria-targeted GFP reporter; and a wild-type *dpy-20* gene rescuing a *dpy-20* mutation in the parental strain. For our purposes here, PD4251 worms constitutively express a strong GFP signal throughout their muscles in the mitochondria.

### Preparation of soil pore water samples and equivalent metal mixtures; metal determinations

Soil samples (~3 kg) from the P79 mine site were collected in 2009 as composite samples (see Rodríguez et al. 2006) made up of 21 increments collected from the upper 25 cm of soil, with a 5 m distance between increments, using an Eijkelpamp soil sampling kit (for further details, see <http://www.eijkelpamp.com/en/products/soil/soil-and-sediment-sampling/soil-sample-ring-kits/sample-ring-kit/sample-ring-kit.htm>). Few if any endogenous nematodes were present. Soil samples (pH 4.5) sent to Nottingham were air-dried at 60°C to constant weight (48–72 h), passed through

a 2 mm sieve to remove debris, and stored dry in sealed containers. Two dried and finely milled control soils were supplied from the soil-sample collection held by the University of Nottingham Environmental Science Division (Sutton Bonington, courtesy of Dr Liz Bailey); one was an arable topsoil (AR) and the other a woodland topsoil (WL). For soil pore water extraction, 60 g samples of dry soil were mixed vigorously using a glass rod with 15 ml of ultrapure water (UW) so as to wet the soil completely, then placed overnight at 4°C in a sealed beaker. This ratio is similar to that used previously with spiked artificial soils to achieve 50% MHC (moisture holding capacity; Power and de Pomerai 1999). The wet soil was split between two 20 ml syringe barrels plugged with a stainless steel mesh disc and glass wool, as described by Power and de Pomerai (1999). To eliminate any possibility of metal contamination from the disc, all metal analyses were repeated on soil pore water extracted using glass wool only. The syringe barrels were placed in 50 ml plastic tubes and centrifuged at  $4,500\times g$  for 10 min at room temperature. In total, 7–8 ml of soil pore water was recovered from each soil sample after 2–4 spins. Soil pore water extracts from each sample were pooled and re-centrifuged at  $10,000\times g$  to remove any remaining sediment. For metal determinations, soil pore water samples were diluted tenfold in a final concentration of 2.5% Analar grade (v/v)  $\text{HNO}_3$ . For extraction of total metals, 2 g of soil was finely ground in an agate ball mill (Retsch model PM400); 200 mg samples were digested with concentrated nitric acid (2 ml) and perchloric acid (1 ml) overnight at 80°C (8 h) and 100°C (2 h), after which 2.5 ml of 70% (v/v) hydrofluoric acid (Trace Element Grade, Fischer Scientific, UK) was added and the digestion continued at 120°C (1 h), 140°C (3 h) and 160°C (4 h) followed by cooling to 50°C. All of these digestion steps were performed in a Teflon-coated graphite block digester (Analysco, UK). Digested samples were diluted to 50 ml using UW ( $18.3 \text{ M}\Omega \text{ cm}^{-1}$ ) and a further tenfold before metal determinations. Multi-element analysis of diluted soil pore water or soil digests was undertaken by ICP-MS (Thermo-Fisher Scientific X-Series<sup>II</sup>) with a hexapole collision cell (7% hydrogen in helium) to remove polyatomic interferences. Samples were introduced from an autosampler (Cetac ASX-520 with  $4 \times 60$ -place sample racks) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific;  $1 \text{ ml min}^{-1}$ ). Internal standards were introduced to the sample stream via a T-piece and included Sc ( $100 \text{ ng ml}^{-1}$ ), Rh ( $20 \text{ ng ml}^{-1}$ ) and Ir ( $10 \text{ ng ml}^{-1}$ ) in 2%  $\text{HNO}_3$ . External multi-element calibration standards (Claritas-PPT grade CLMS-2, Certiprep/Fisher) included Al, As, Ba, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Rb, Se, Sr, U, V and Zn, in the range 0–100  $\mu\text{g l}^{-1}$ . Unfortunately, due to its volatility, Hg could not be measured using this equipment. Sample

processing used Plasmalab software (version 2.5.4; Thermo-Fisher Scientific) set to employ separate calibration blocks and internal cross-calibration. The metals reported here are Al, As, Ba, Cd, Cr, Cu, Fe, Mn, Ni, Pb, U, V and Zn (Table 3). Equivalent mixtures of the major metal ions present were prepared at the same final concentrations using chloride salts, apart from  $\text{Pb}^{2+}$  as nitrate and  $\text{As}^{3+}$  as sodium arsenite; all metal salts were Analar grade from BDH Ltd (Poole, Dorset, UK). For the mine soil–water sample (P79), simplified metal mixtures containing the most predominant toxic metal ions were prepared and tested at tenfold *lower* concentrations than those present in the original P79 soil water (which was also tested at the same tenfold dilution, to avoid worm lethality): Zn contains only  $\text{Zn}^{2+}$  ( $500 \text{ mg l}^{-1}$ ); 3 M contains the same concentration of  $\text{Zn}^{2+}$  plus  $\text{Cu}^{2+}$  ( $2.0 \text{ mg l}^{-1}$ ) and  $\text{Cd}^{2+}$  ( $1.7 \text{ mg l}^{-1}$ ); 5 M also contains  $\text{Al}^{3+}$  ( $89 \text{ mg l}^{-1}$ ) and  $\text{Mn}^{2+}$  ( $23 \text{ mg l}^{-1}$ ); lastly, 8 M contains these 5 plus  $\text{Fe}^{3+}$  ( $0.25 \text{ mg l}^{-1}$ ),  $\text{Ni}^{2+}$  ( $0.5 \text{ mg l}^{-1}$ ) and  $\text{Pb}^{2+}$  ( $0.2 \text{ mg l}^{-1}$ ).

#### GFP reporter assays

Transgenic worms were grown at 15°C on standard nematode growth medium (NGM) agar plates pre-seeded with a lawn of *lac*-deleted *E. coli* (strain P90C) as food bacteria (Dennis et al. 1997). Mixed cultures containing all developmental stages of *C. elegans* were washed off NGM plates with ice-cold K medium (53 mM NaCl, 32 mM KCl; Williams and Dusenbery 1990); worms were allowed to settle 2–3 times on ice for  $\sim 15$  min, and resuspended in fresh K medium to remove bacteria. Pellets were resuspended in 1–2 ml of K medium, and worm numbers were counted in 5  $\mu\text{l}$  aliquots under a low-power inverted microscope. After dilution as required, the worm suspension was stirred gently whilst adding 50  $\mu\text{l}$  aliquots (containing 500–1,000 worms) to 250  $\mu\text{l}$  samples of soil pore water or metal solutions or mixtures in all wells of a 24-well plate (Nunc Ltd); quadruplicate independent tests were run in different plates for each strain. For example, in one experiment the mean number of worms was  $53 \pm 6$  (SEM) across 6 replica 5  $\mu\text{l}$  aliquots; given greater accuracy in measuring larger volumes (50  $\mu\text{l}$ ), the actual number of worms added to each assay well should vary by no more than 10% from the mean (530). Parallel negative controls were also prepared, with UW replacing soil pore water or metals. Dose–response patterns have been determined for all 24 test genes for  $\text{Zn}^{2+}$  and for 10 of the metals analysed in the soil water mixture (omitting Ba, U and V), though detailed response data is presented here for only the predominant metal present, namely zinc. All other reporter responses are presented as Supplementary Material. In addition, we have tested simple 2-component metal mixtures, using much lower doses of each ion (20% of those



eliciting the strongest observed induction), both singly and combined: namely 2 mg l<sup>-1</sup> for Hg<sup>2+</sup>, 5 mg l<sup>-1</sup> for Cd<sup>2+</sup>, and 20 mg l<sup>-1</sup> for Fe<sup>3+</sup>. All exposures were at 20°C in sealed humidified plastic boxes so as to minimise evaporation. At 4–6 h (early), 8–16 h (intermediate) and 20–30 h (late) time points, the contents of each well were transferred to a non-fluorescent black U-bottomed 96-well micro-plate (Nunc Ltd) and kept 15 min on ice to let worms settle. GFP fluorescence in worms was measured in a Perkin-Elmer Victor 1420 Multilabel plate reader with narrow bandpass filters for excitation (485 nm) and emission (525 nm). After four GFP measurements at the base of each well, its contents were transferred back into the 24-well source plate and exposure was continued until the next reading, so generating time-course data from a single set of assays. All 24-well plates were routinely checked at the end of the exposure period (24–30 h) to look for immobile (paralysed or dead) worms. In cases where the prevalence of such worms exceeded 20% (as happened initially with 100 mg l<sup>-1</sup> of Cr<sup>6+</sup>), we have discounted the results and re-run the experiment with a lower maximum dose (40 mg l<sup>-1</sup> for Cr<sup>6+</sup>; Table 2). Widespread lethality also led in most cases to a sharp drop in the GFP fluorescence recorded. Strain PD4251 (*myo-3::GFP*) showed atypically high lethality at 100 mg l<sup>-1</sup> Fe<sup>3+</sup> (>50%) and some increase in lethality (~25%) at 100 mg ml<sup>-1</sup> of both Al<sup>3+</sup> and As<sup>3+</sup>; the tests shown in Table 5 therefore use only 25 mg ml<sup>-1</sup> of Fe<sup>3+</sup> and 50 mg ml<sup>-1</sup> each of Al<sup>3+</sup> and As<sup>3+</sup>.

### Statistical analysis

For dose response data, mean fluorescence measurements (in arbitrary relative fluorescence units, RFU ± SEM) were analysed by one-way ANOVA with Dunnett's post hoc multiple comparisons test against zero controls (no metal) at the same time point. In Figs. 2 and 3, as well as in Tables 1, 4, 5 and the Supplementary Material, all fluorescence measurements have been normalised against the corresponding zero (UW) controls at the same time point, giving an expression ratio which is always 1.0 for the UW controls. Other comparisons within data sets were made using one-way ANOVA with Bonferroni's post hoc multiple comparisons test. Where each test condition was compared against an individual UW control, Student's *t* test was used (Table 5 only).

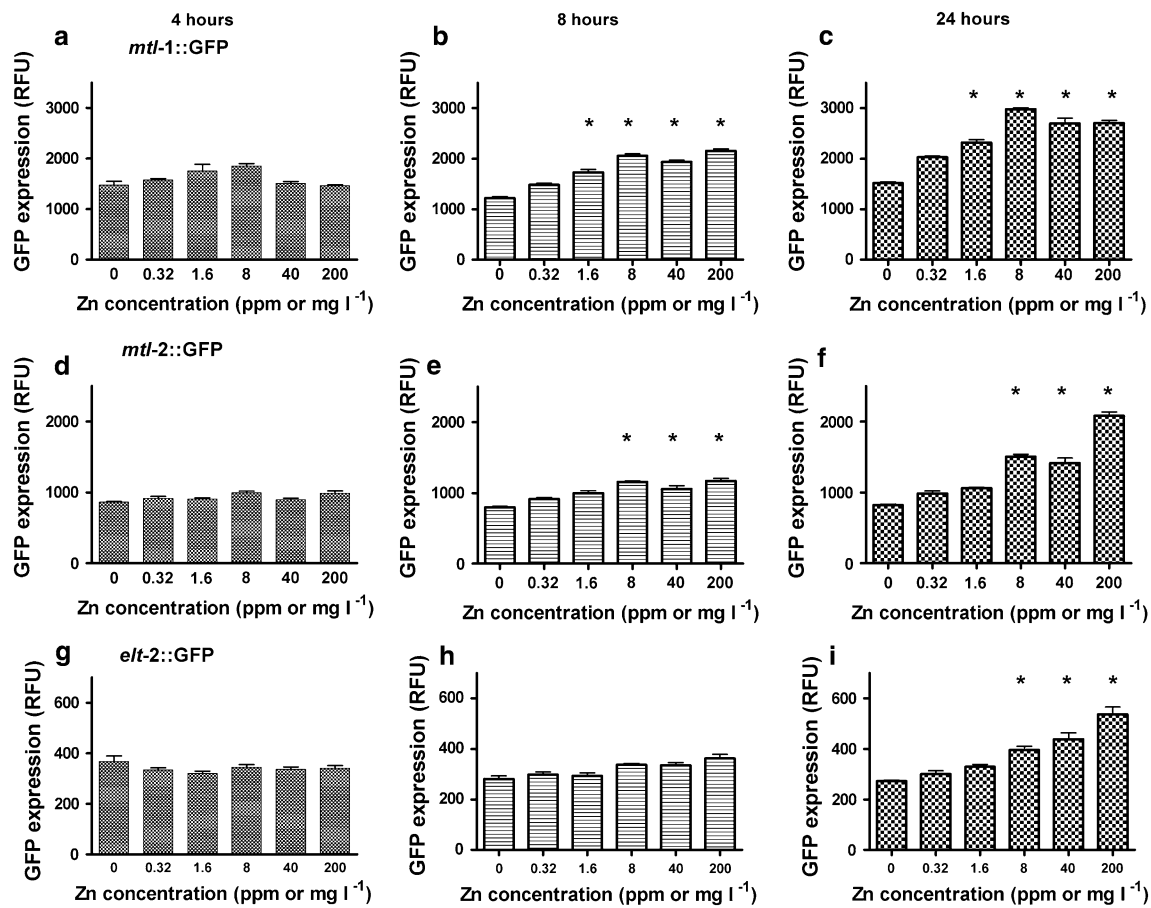
## Results

Typical examples of transgene responses to zinc are shown in Fig. 1 for the two genes encoding metal-binding *mtls* (*mtl-1/-2*) and their principal transcriptional regulator encoded by *elt-2* (Moilanen et al. 1999). All 3 genes show

modest zinc inducibility at 8 and 24 h, though little response is evident at 4 h. This result is in agreement with previous studies indicating that *mtl-2::GFP* is only weakly inducible by Zn (e.g. ~twofold at 130 mg l<sup>-1</sup> after 24 h in the study by Ma et al. 2009a). Interestingly, the *mtl-1* gene shows maximal (~twofold) induction at an intermediate dose of 8 mg l<sup>-1</sup> (Fig. 1c), whereas both *mtl-2* and *elt-2* show greater induction (>twofold) at the highest test dose of 200 mg l<sup>-1</sup> (Fig. 1f, i). As shown in the first row of Table 2 (below), the published 24-h LC50 for Zn is only 202 mg l<sup>-1</sup> (Williams and Dusenbery 1990); however, in the absence of bacteria (as here), the same group reported a substantially higher 24 h LC50 of 425 mg l<sup>-1</sup> (Tatara et al. 1998). We found that even 500 mg l<sup>-1</sup> Zn causes <10% mortality among our worms (all strains) after 24 h (data not shown). RNA interference experiments (unpublished data) confirm a key role for the ELT-2 GATA TF in metal regulation of *mtl-1*, although our evidence is weaker for *mtl-2* (data not shown); this suggests that other uncharacterised TFs may assist in metal regulation of this gene. Both *mtl* genes are much more strongly inducible by the other Group IIB metals, Cd and Hg (nearly fivefold induction at 10 mg l<sup>-1</sup> Hg<sup>2+</sup>; see Supplementary Material), consistent with their much greater toxicity as compared to Zn<sup>2+</sup>.

Our current data-set includes 11 different metals and 24 different stress-response genes, so presenting these data in the same format as Fig. 1 would be lengthy and cumbersome. One way of summarising these data is to tabulate expression ratios (metal-treated/control), so that each time-point can be reduced to a set of ratios for each test dose (where the control is always 1.0). Table 1 presents an abbreviated summary of similar data for the effects of zinc on all 24 test genes included in our panel of GFP reporters. For simplicity, and since very few expression changes were observed at the early time point (4–6 h), expression ratios are shown only for the intermediate (8–16 h) and late time points (20–30 h). Many test genes are also induced by ~twofold at the highest dose tested (200 mg l<sup>-1</sup>), but curiously the two strongest zinc inductions (>threefold) are for classic xenobiotic stress-response genes, namely the *gst-1* and *cyp-35A2* genes, respectively encoding a glutathione-S-transferase and a strongly inducible cytochrome P450 (Menzel et al. 2001).

The first row of Table 2 shows published LC50 values for all 11 metals, derived from the data of Williams and Dusenbery (1990; bacteria present) and Tatara et al. (1998; no bacteria). A direct comparison between these two studies is provided for Zn and Cd, suggesting that LC50 values may be higher in the absence of bacteria (as in this study), possibly because ingestion of food increases metal intake via the gut. With this in mind, our highest test doses for most metals are below the LC50, with the major exception of Fe. In our hands, worms of all strains can



**Fig. 1** Zinc responsiveness of the *mtl*-related genes in *C. elegans*. GFP reporter expression was measured for each transgene (see “Materials and Methods” section) in 4 replicates for each of 5 Zn doses (0.32–200 mg l<sup>-1</sup>) plus a zero UW control (no zinc). Each panel shows the mean and SEM from 4 determinations at the

indicated time point (4 h in left-hand panels; 8 h in centre panels; 24 h in right-hand panels). Top row, *mtl-1::GFP* responses; centre row, *mtl-2::GFP* responses; bottom row, *elt-2::GFP* responses. \**p* ≤ 0.05 (Dunnett’s test)

tolerate concentrations of >100 mg l<sup>-1</sup> Fe<sup>3+</sup> without excessive mortality (<20% immobile after 24 h; see “Materials and Methods” section). Since Tatara et al. (1998) also tested Fe<sup>3+</sup> (but as nitrate rather than chloride) and found a low 24 h LC50 of only 18 mg l<sup>-1</sup>, we can only tentatively suggest possible differences in metal speciation as an explanation for this anomaly. In our experience, the transgenic strains used here differ little from wild-type worms (Bristol N2 strain) in terms of their sensitivity to different toxicants, as shown by Ma et al. (2009a) for an *mtl-2::GFP* transgenic strain. Despite the presence of a transgene array, the wild-type gene function remains intact, though TF sequestration onto the array remains a formal possibility. Many published studies on metal toxicity in *C. elegans* have measured sublethal endpoints such as reproduction, lifespan, motility and feeding inhibition (e.g. Jones and Candido 1999; Swain et al. 2004; Anderson et al. 2001), but none of them covers all the metals tested here, and therefore these endpoints are not further explored or discussed.

The remainder of Table 2 provides a brief overview of the effects of all 11 metal toxicants on transgene expression, indicating only the maximal response observed for that metal (irrespective of time point or test dose). The full data-set (for all genes, metals, time-points and test doses) is available as Supplementary Material. In Table 2, the extent of up- or down-regulation (rounded to 1 decimal place) is categorised into 4 bands: >1.5 fold down (–); 1.5–1.9 fold up (+); 2–2.9 fold up (++); and >threefold up (+++). Most of the toxic divalent metals tested (Cu, Cd, Hg and Zn) induce the heat-shock genes quite strongly, but their principal TF (encoded by the *hsf-1* gene) shows weaker induction. Similar patterns of response are evident for the metal-response group of genes (though Cd does not up-regulate *elt-2*) and for the xenobiotic response genes (3 *cyp* and 2 *gst* genes, all known to be stress-inducible). By contrast, the only genes consistently induced by these metals among the oxidative stress group are *sod-4* (encoding an extracellular superoxide dismutase) and C11E4.1 (an uncurated gene encoding a gut-expressed

**Table 1** Expression of stress-related GFP reporter transgenes following zinc exposure

Gene	Zn dose											
	GFP expression ratio at intermediate time point (8 h)						GFP expression ratio at late time point (24 h)					
	0 mg l <sup>-1</sup>	0.32 mg l <sup>-1</sup>	1.6 mg l <sup>-1</sup>	8.0 mg l <sup>-1</sup>	40 mg l <sup>-1</sup>	200 mg l <sup>-1</sup>	0 mg l <sup>-1</sup>	0.32 mg l <sup>-1</sup>	1.6 mg l <sup>-1</sup>	8.0 mg l <sup>-1</sup>	40 mg l <sup>-1</sup>	200 mg l <sup>-1</sup>
<i>mtl-1</i>	1.00	1.21	1.41	<b>1.68</b>	<b>1.58</b>	<b>1.76</b>	1.00	1.46	1.57	<b>2.09</b>	<b>1.97</b>	<b>1.86</b>
<i>mtl-2</i>	1.00	1.14	1.25	<b>1.45</b>	1.32	<b>1.46</b>	1.00	1.20	1.29	<b>1.83</b>	<b>1.72</b>	<b>2.53</b>
<i>elt-2</i>	1.00	1.06	1.04	1.20	1.19	1.29	1.00	1.10	1.21	<b>1.45</b>	<b>1.61</b>	<b>1.97</b>
<i>hsp-16.1</i>	1.00	1.10	1.15	<b>1.66</b>	<b>1.86</b>	<b>2.30</b>	1.00	1.32	1.39	<b>1.69</b>	<b>1.91</b>	<b>2.38</b>
<i>hsp-16.2</i>	1.00	1.19	1.22	1.29	<b>1.49</b>	<b>2.33</b>	1.00	1.08	1.09	1.09	1.32	<b>2.43</b>
<i>hsp-3</i>	1.00	1.29	1.23	1.29	1.32	<b>1.68</b>	1.00	1.08	1.06	1.15	1.24	<b>1.49</b>
<i>hsp-6</i>	1.00	1.22	1.21	1.23	1.18	<b>1.71</b>	1.00	1.32	1.30	1.25	<b>1.45</b>	<b>1.93</b>
<i>hsp-60</i>	1.00	1.13	1.10	1.16	1.27	<b>2.40</b>	1.00	1.17	1.17	1.18	1.23	<b>2.61</b>
<i>hsp-70</i> C12C8.1	1.00	1.10	1.13	1.10	1.24	<b>1.83</b>	1.00	1.01	1.00	1.01	1.22	<b>1.47</b>
<i>hsf-1</i>	1.00	1.06	1.10	1.11	1.30	<b>1.80</b>	1.00	1.00	1.05	1.04	1.10	<b>1.61</b>
<i>gst-1</i>	1.00	1.38	1.53	1.54	1.57	<b>1.75</b>	1.00	<b>1.81</b>	<b>2.35</b>	<b>2.79</b>	<b>2.90</b>	<b>3.46</b>
<i>gst-4</i>	1.00	1.14	1.15	1.12	1.04	<b>1.46</b>	1.00	<b>1.59</b>	<b>1.62</b>	<b>1.50</b>	1.31	<b>1.91</b>
<i>cyp-29A2</i>	1.00	0.99	0.99	1.03	1.40	<b>2.36</b>	1.00	1.05	1.09	1.16	<b>1.60</b>	<b>2.45</b>
<i>cyp-34A9</i>	1.00	1.18	1.35	<b>1.47</b>	<b>1.64</b>	<b>1.87</b>	1.00	1.11	1.27	1.41	1.39	<b>2.06</b>
<i>cyp-35A2</i>	1.00	1.15	1.35	<b>1.76</b>	<b>2.04</b>	<b>2.48</b>	1.00	1.33	<b>1.74</b>	<b>2.54</b>	<b>2.76</b>	<b>3.81</b>
<i>sod-1</i>	1.00	1.03	1.05	1.30	1.18	1.31	1.00	1.06	1.09	1.31	1.29	<b>1.89</b>
<i>sod-3</i>	1.00	1.05	0.97	0.80	<i>0.67</i>	0.70	1.00	1.21	1.14	1.08	0.93	0.86
<i>sod-4</i>	1.00	1.07	1.18	1.32	<b>1.72</b>	<b>2.13</b>	1.00	1.15	1.27	1.43	<b>1.71</b>	<b>2.53</b>
<i>ctl-2</i>	1.00	1.13	1.14	1.25	1.26	<b>1.48</b>	1.00	1.21	1.21	1.34	1.32	<b>1.92</b>
gpA T09A12.2	1.00	1.10	1.19	1.22	1.19	1.27	1.00	1.20	1.44	<b>1.64</b>	<b>1.60</b>	<b>2.25</b>
gpB C11E4.1	1.00	1.11	1.20	1.22	1.17	1.36	1.00	1.19	1.30	<b>1.45</b>	<b>1.46</b>	<b>2.15</b>
<i>skn-1</i>	1.00	1.08	1.14	1.22	1.32	<b>1.48</b>	1.00	1.14	1.16	1.30	1.36	<b>1.88</b>
<i>cep-1</i>	1.00	1.42	1.18	1.14	1.18	1.37	1.00	<b>1.51</b>	<b>1.56</b>	<b>1.56</b>	<b>2.02</b>	<b>2.72</b>
<i>daf-16</i>	1.00	1.21	1.25	1.24	1.39	1.31	1.00	1.33	1.44	<b>1.47</b>	<b>1.67</b>	<b>1.78</b>

GFP expression was measured as in Fig. 1 (see “Materials and Methods” section), but all results are expressed as ratios between GFP expression at each Zn<sup>2+</sup> dose and in the corresponding zero (UW) controls (n = 4). Thus the expression ratio for all UW controls is 1.0. Statistically significant differences in expression of >1.5-fold (rounded) are highlighted in bold and those >twofold are also underlined. Early responses at 4 h have been omitted, since few genes show any significant change in expression at this stage. HSP70 designates gene C12C8.1, gpA designates gene T09A12.2, and gpB designates gene C11E4.1. A single instance of 1.5-fold down-regulation (for *sod-3*) is shown in italics

glutathione peroxidase, here designated gpB). The stress-related TFs encoded by the *daf-16* and *cep-1* (p53) genes show inconsistent patterns of inducibility. Among the other metals tested, trivalent Al<sup>3+</sup>, Fe<sup>3+</sup> and As<sup>3+</sup> (as arsenite) show broadly similar patterns of stress-gene induction to the divalent metals, except that there is usually much less effect on the metal response group (apart from Al<sup>3+</sup>). Somewhat different response patterns are seen for Cr<sup>6+</sup>, which unusually activates *hsp-70::GFP* reporter expression more strongly than the *hsp-16.1/16.2* genes (cf. Cu), along with the *gst-1* and *cyp-35A2* genes (cf. Zn<sup>2+</sup>). Divalent Mn<sup>2+</sup> shows almost no effect on any transgene at doses up to 200 mg l<sup>-1</sup>, and Pb<sup>2+</sup> (up to 50 mg l<sup>-1</sup>) has only small

effects on *cyp-34A9* and possibly *mtl-1*. Ni<sup>2+</sup> (up to 200 mg l<sup>-1</sup>) induces the *hsp-16.1/0.2* genes strongly, along with *cyp-34A9*.

We have developed mathematical models for several regulatory gene circuits involved in the overall network of stress responses, including *daf-16* and the heat-shock genes (not further discussed). One of these models is based on the activation of *mtl* genes in the intestine by the ubiquitous gut TF ELT-2. Normally this is prevented by an unknown metal-sensitive repressor (*U*), but inducing metal ions (usually divalent) relieve this repression by binding to *U*, allowing ELT-2 to switch on *mtl* expression (Moilanen et al. 1999). The feedback loop is closed when MTL

**Table 2** Summary of strongest responses for 11 metals in 24 GFP transgenic strains

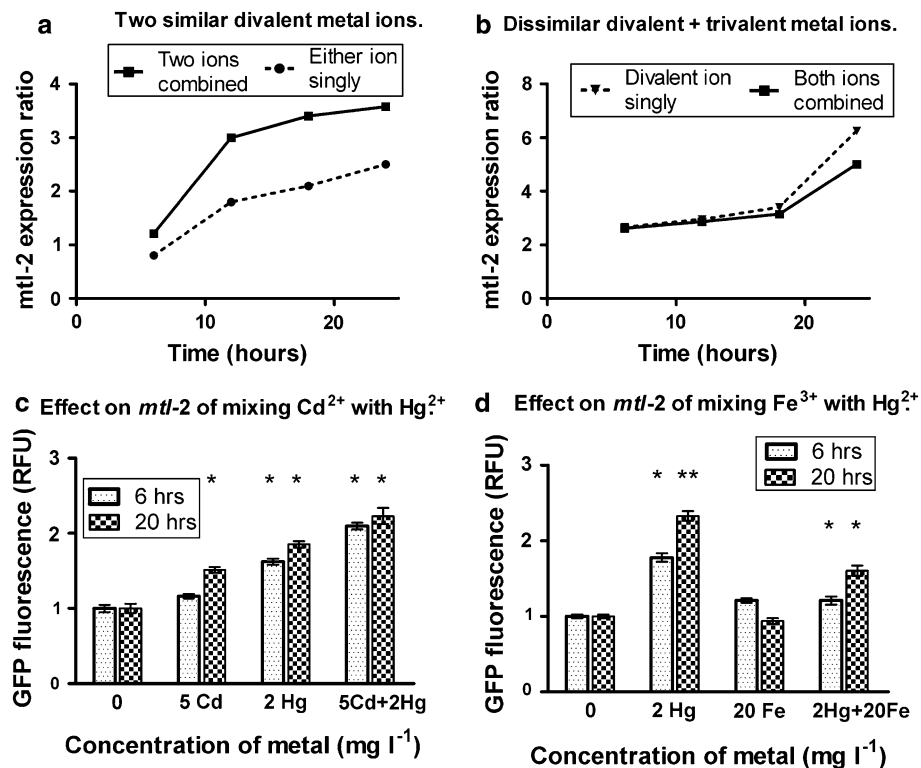
Dose range:	Meta										
	Zn <sup>2+</sup> 0–200 mg l <sup>-1</sup>	Cd <sup>2+</sup> 0–22 mg l <sup>-1</sup>	Hg <sup>2+</sup> 0–10 mg l <sup>-1</sup>	Cu <sup>2+</sup> 0–15 mg l <sup>-1</sup>	Fe <sup>3+</sup> 0–100 mg l <sup>-1</sup>	Cr <sup>6+</sup> 0–40 mg l <sup>-1</sup>	As <sup>3+</sup> 0–100 mg l <sup>-1</sup>	Al <sup>3+</sup> 0–100 mg l <sup>-1</sup>	Ni <sup>2+</sup> 0–200 mg l <sup>-1</sup>	Mn <sup>2+</sup> 0–200 mg l <sup>-1</sup>	Pb <sup>2+</sup> 0–50 mg l <sup>-1</sup>
Mean 24 h LC50 (see legend) in mg l <sup>-1</sup>	202	904	10	22	<u>18</u>	156	182	79	2916	<u>7260</u>	129
	<u>425</u>	<u>1307</u>									
Heat-shock (proteotoxicity response) genes											
<i>hsp</i> -16.1	++	+++	++	+	+++	+	++	+	+++		
<i>hsp</i> -16.2	++	++	+	+++	+++	+	+++		++		
<i>hsp</i> -3	+	+++	+	+++	+			++	(+)		
<i>hsp</i> -6	+	++	++	++	+	+	++	++			
<i>hsp</i> -60	++	++	+	+++		++	++	++		(+)	
<i>hsp</i> -70	+	+	+	++		++	+	+++			
C12C8.1											
<i>hsf</i> -1	+	+	+	+	–			+			
Metal response genes											
<i>mtl</i> -1	++	+	+	++			+		+		(+)
<i>mtl</i> -2	++	+++	+++	+	++		+	++	(+)		
<i>elt</i> -2	++		+	++		++					
Xenobiotic response genes											
<i>gst</i> -1	+++	+	+	++	–	+	++	+++			
<i>gst</i> -4	+	++	+	++		++	+	+++	+		
<i>cyp</i> -29A2	++	++	+	++			+	++			
<i>cyp</i> -34A9	++	++	+	+	+		++	+	++		+
<i>cyp</i> -35A2	+++	+	++	++	+	+	++	++		–	
Oxidative stress response genes											
<i>sod</i> -1	+		+	+		++			+		
<i>sod</i> -3	–		+	++							
<i>sod</i> -4	++	++	++		+++	+	+				
<i>ctl</i> -2	+		+		–						
gpA	++		+	++	++			++			
T09A12.2											
gpB	++	+	+	++	++		++				
C11E4.1											
<i>skn</i> -1	+		+					–			
Core stress-responsive TFs											
<i>cep</i> -1	++		+	+		+	++			–	
<i>daf</i> -16	+				++			+	+		

This table summarises the maximal changes in expression pattern seen with each metal for all 24 transgenes; all expression ratios have been normalised against the corresponding UW control as 1.0. Complete dose–response data (in a similar format to Table 1) can be found in the Supplementary Material. Expression ratio (ER) between 0.67 and 1.44, no entry; ER <0.66, –; ER >1.45 but <1.94, +; ER >1.95 but <2.94, ++; ER >2.95, +++. For Ni, Pb and Mn, statistically significant up-regulation with ER >1.25 but <1.44 is shown as (+). The *top row* shows mean 24-h LC50 data for each metal taken from Williams and Dusenbery (1990), apart from the underlined entries which are from Tatara et al. (1998)

proteins sequester the toxic metal ions, so switching off the *mtl* genes as *U* dissociates from the metal and is freed to block the action of ELT-2. Our mathematical formulation of this genetic circuit will be published separately (Haque

2011), but its main predictions in relation to simple two-component metal mixtures are summarised in the upper panels of Fig. 2. Figure 2a predicts that chemically similar (e.g. divalent) metal ions are likely to show additive effects





**Fig. 2** Modelling predictions and validation of simple metal mixture effects on *mtl-2* expression. Key predictions of our mathematical model for the *mtl* gene-circuit (see text) are shown in **a** for similar divalent metal-ion inducers (additive effects) and in **b** for dissimilar metal ions (where interfering effects are predicted). **c** and **d** Broadly confirm these predictions in laboratory tests for *mtl-2::GFP* induction by simple metal mixtures, using  $\text{Hg}^{2+}$  with  $\text{Cd}^{2+}$  (chemically similar strong inducers) in **c**, and  $\text{Hg}^{2+}$  with  $\text{Fe}^{3+}$  (chemically dissimilar) in

**d**. In both parts, the concentrations of metals tested (both singly and in mixtures) are one-fifth of the highest test dose (giving strong induction of the *mtl-2* transgene). Data are shown at both 6 h (stippled) and 20 h (chequered). Significant changes in GFP expression are indicated by asterisks: \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ . 5 Cd = 5  $\text{mg l}^{-1}$  cadmium ( $\text{Cd}^{2+}$ ); 2 Hg = 2  $\text{mg l}^{-1}$  mercury ( $\text{Hg}^{2+}$ ); 20 Fe = 20  $\text{mg l}^{-1}$  ferric iron ( $\text{Fe}^{3+}$ )

on the level of *mtl* gene expression, and moreover the magnitude of this additive effect should not change greatly over time. By contrast, Fig. 2b predicts that metals which are chemically dissimilar (e.g. where one is a much better inducer, and/or where the other cannot be sequestered effectively via MTL proteins) are likely to show interfering effects, whose magnitude should increase over time. These predictions have been verified in the laboratory, as illustrated in the lower panels of Fig. 2. At one-fifth of our highest test doses, the similar divalent ions  $\text{Cd}^{2+}$  and  $\text{Hg}^{2+}$  (both strong *mtl* inducers) show a clear additive effect on *mtl-2::GFP* expression in Fig. 2c, and furthermore the magnitude of this effect is very similar both at 6 and at 20 h. The differences between the Cd + Hg mixture and either Cd or Hg alone are significant (Bonferroni test:  $p \leq 0.05$ ) at both time points, although the effect of Cd alone is not significantly different from the zero control at 6 h. In Fig. 2d, again at one-fifth of our highest test doses, the divalent  $\text{Hg}^{2+}$  ion is combined with the trivalent ferric ( $\text{Fe}^{3+}$ ) ion, which is a much weaker inducer, and is likely to be sequestered as ferritin rather than through binding to

MTLs. For this dissimilar metal mixture, there is a slight but significant ( $p < 0.05$ ) reduction in *mtl-2::GFP* reporter expression at 6 h (as compared to  $\text{Hg}^{2+}$  alone) but a much greater reduction at 20 h ( $p < 0.01$ ). Note that this low dose of  $\text{Fe}^{3+}$  alone has no significant effect on reporter expression at either time point ( $p > 0.05$ ). These and other mixture experiments (involving Al, Cu and Zn; data not shown) confirm the main predictions of our mathematical model in relation to metal mixture effects on *mtl* gene expression.

Turning to the soil pore water studies, Table 3 shows the metal concentrations detected by ICP-MS in the P79 mine soil as well as in samples of pore water from soil at this abandoned mine site in the Murcia area of Spain, and from both AR and WL topsoils as controls (means of 1–4 determinations). Two sets of results are shown for the mine-derived soil pore water (P79), prepared from independent soil extractions carried out 6 months apart; the second differs from the first by omitting the metal disc during centrifugation, but this did not reduce the high metal concentrations measured subsequently. Both soil pore

**Table 3** Metal contents of soil pore-water samples and of total soil from the P79 mine site

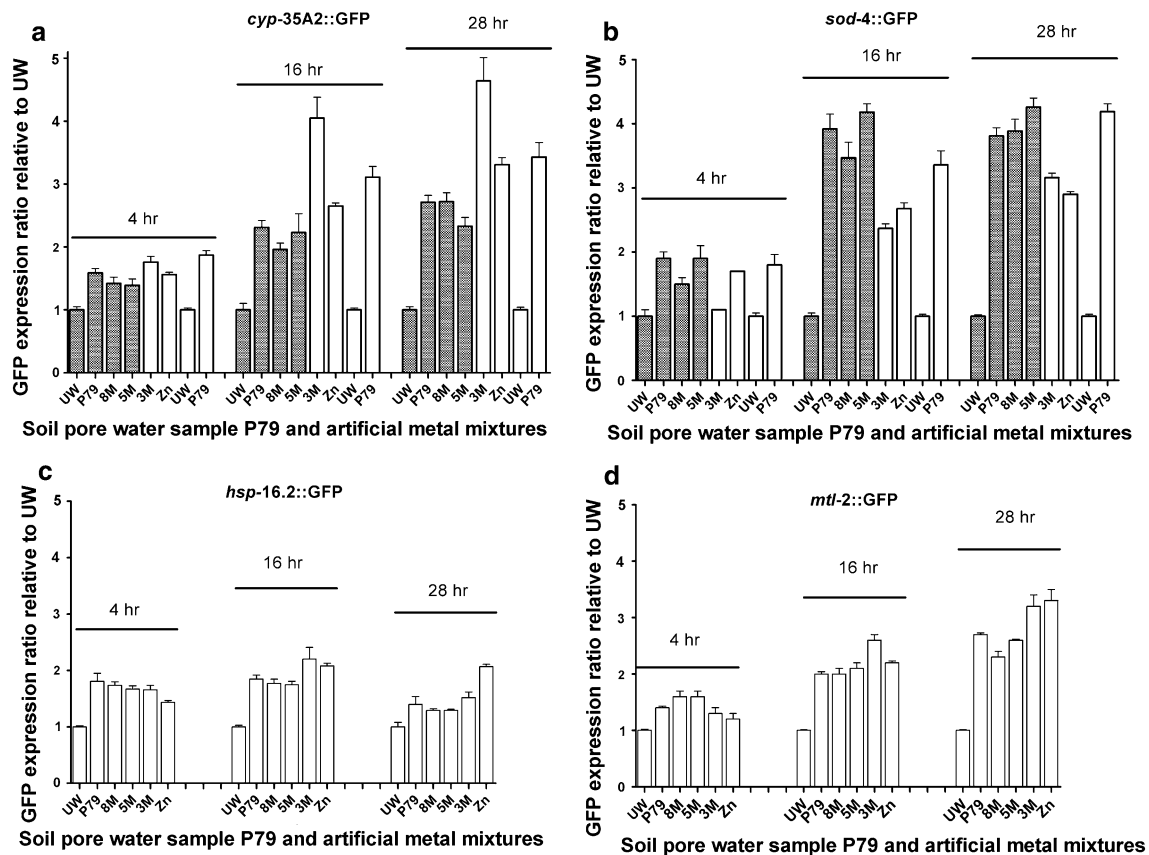
Metals													
Al	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Cd	Ba	Pb	U	
P79 soil water n = 2 (mg l <sup>-1</sup> )	891.3 ± 1.0	0.0014 ± 0.0004	0.076 ± 0.00051	235.8 ± 4.0	2.192 ± 0.5	4.639 ± 0.024	21.240 ± 0.07	5016 ± 13.5	0.027 ± 0.0023	17.72 ± 0.2	0.036 ± 0.0012	2.234 ± 0.106	0.286 ± 0.0006
P79 soil water reassay, n = 4 (mg l <sup>-1</sup> )	ND	0.0034 ± 0.0007	0.054 ± 0.0076	210.15 ± 24.5	2.87 ± 0.62	5.03 ± 0.6	15.74 ± 2.1	4925 ± 554	0.016 ± 0.0022	16.04 ± 1.92	ND	1.89 ± 0.34	0.189 ± 0.024
AR arable topsoil, n = 2 (mg l <sup>-1</sup> )	0.76 ± 0.05	0.012 ± 0.001	0.007 ± 0.0002	0.37 ± 0.02	0.72 ± 0.025	0.025 ± 0.00015	0.30 ± 0.015	0.60 ± 0.032	0.011 ± 0.001	0.002 ± 0.0001	ND	0.029 ± 0.002	0.00004 ± 0.000004
WWL woodland topsoil, n = 1 (mg l <sup>-1</sup> )	ND	0.131	0.131	1.99	3.85	0.83	0.14	1.21	0.27	0.00002	ND	0.11	0.0005
P79 total soil n = 3 (mg kg <sup>-1</sup> )	ND	63.91 ± 1.96	49.04 ± 2.01	406.3 ± 31.0	58612 ± 1858	16.56 ± 3.58	215.2 ± 7.74	6956 ± 306	208.7 ± 7.64	14.37 ± 0.672	ND	10813 ± 315	9.22 ± 0.35

Metal analysis of soil pore water from the P79 site was performed twice, the second time omitting the metal mesh disc during centrifugation (as described in “Materials and Methods” section). Metal analyses were similarly performed on pore water from both the AR and WL control samples. Total metals were also extracted from P79 soil (only) by hydrofluoric acid digestion (as described in “Materials and Methods” section), followed by metal analysis as above. Table 3 shows means and standard deviations (mg l<sup>-1</sup> in soil pore water or mg kg<sup>-1</sup> in soil) derived from 1 to 4 replicate samples; figures are shown for undiluted samples in all cases

ND not determined

water samples show extremely high concentrations of dissolved Zn (4,900–5,000 mg l<sup>-1</sup>), but other metals such as Al (890 mg l<sup>-1</sup>), Mn (210–230 mg l<sup>-1</sup>), Cu (16–21 mg l<sup>-1</sup>), Cd (16–18 mg l<sup>-1</sup>), Ni (5 mg l<sup>-1</sup>) and Pb (2 mg l<sup>-1</sup>) are also present in large amounts. Unfortunately, due to its volatility, Hg concentrations could not be measured. Other metals measured, but not listed in Table 3, include Na (125 mg l<sup>-1</sup> in P79 soil pore water), Mg (1,450 mg l<sup>-1</sup>), K<sup>+</sup> (6.5 mg l<sup>-1</sup>) and Ca (435 mg l<sup>-1</sup>). Also included in Table 3 are the contents of most of these metals in total P79 soil: as expected, by far the most prominent of these are Fe (58,610 mg kg<sup>-1</sup>), Pb (10,810 mg kg<sup>-1</sup>) and Zn (6,960 mg kg<sup>-1</sup>). Comparing these data sets implies that a substantial proportion (<20%) of the total Zn, Cd and Mn in P79 soil (pH 4.5) is water-soluble. By contrast, neither AR nor WL topsoil samples contains large amounts of dissolved metals in their pore water; the only concentrations >1 mg l<sup>-1</sup> are Mn (1.99), Fe (3.85; higher than P79!) and Zn (1.21) in the WL sample, while only Fe (0.72) and Zn (0.60) approach this level in AR.

Figure 3 compares tenfold dilutions of soil pore water from the P79 mine site against four increasingly simplified artificial metal mixtures (8, 5, 3 M and Zn—compositions as specified in “Materials and Methods” section). Figure 3 shows typical response patterns for 4 strongly induced stress-reporter transgenes (Tables 1, 2), namely: *cyp-35A2* (part a), *sod-4* (part b), *hsp-16.2* (part c) and *mtl-2* (part d). The first two of these were exposed in 2 batches, each including an UW control and a tenfold dilution of P79 soil pore water; one batch also included the 8 and 5 M artificial metal mixtures, whilst the other included the 3 M and Zn tests. For both *cyp-35A2* and *sod-4*, responses to diluted P79 were broadly comparable between these two batches, after normalising against the UW control as 1.0 (Fig. 3a and b). The other two test genes (*hsp-16.2* and *mtl-2*) were assayed for all 6 test conditions simultaneously, and hence include only a single set of UW and P79 controls (Fig. 2c and d). The three groups of bars in each panel distinguish early (4 h), intermediate (16 h) and late (28 h) responses. For *cyp-35A2*, the effects of the 5 and 8 M mixtures are similar to those of diluted P79 soil water ( $p > 0.05$ , Bonferroni test), implying that the many other metals present in smaller amounts in P79 are largely irrelevant to its effect on the expression of this gene. By contrast the 3 M mixture (comprising divalent metals only) shows a significantly stronger effect than P79 ( $p < 0.01$ ) after 16 or 28 h, whereas the effect of Zn alone is similar to that of P79 ( $p > 0.05$ ). Since 5 and 8 M responses do not differ significantly from P79, this implies that the 3 M response would also exceed those for 5 and 8 M in a direct comparison (unfortunately this was not possible because it straddles two different data-sets). In this instance, the presence of trivalent metals might interfere with the effect



**Fig. 3** Responses of 4 GFP reporter genes to P79 mine soil pore water and artificial metal mixtures. Air-dried soil from the P79 mine site was mixed with 20% w/w UW and soil pore water extracted centrifugally as described in “Materials and Methods” section. Because of its extreme toxicity ( $5,000 \text{ mg l}^{-1} \text{ Zn}$ ), this pore water was diluted tenfold for all tests. This experiment also tested artificial mixtures containing equivalent concentrations of zinc alone (i.e.  $500 \text{ mg l}^{-1} \text{ Zn}$ ) and of the 3, 5 or 8 most prevalent toxic metal ions present in P79 soil water (Table 3), as described in “Materials and Methods” section. All data (mean  $\pm$  SEM,  $n = 4$ ) were normalised relative to the UW controls (expression ratio of 1.0). For parts **a** (*cyp-35A2*) and **b** (*sod-4*), the exposures were conducted in two separate batches (each with its own UW and P79 controls), distinguished by

shaded versus open bars. For **c** (*hsp-16.2*) and **d** (*mtl-2*), all comparisons were made within a single experiment. For **a** and **b**, each group of 8 bars shows (from left to right): UW control, P79 ( $\times 10$  diluted), 8 and 5 M metal mixes (batch 1, shaded), followed by 3 M metal mix and Zn alone plus UW control and P79 ( $\times 10$  diluted) (batch 2, open). In **c** and **d**, each group of 6 bars shows (left to right): UW control, P79 ( $\times 10$  diluted), 8, 5, 3 M metal mixes, and Zn alone. 4 h responses are on the left, 16 h in the centre and 28 h on the right. The  $p$  values obtained from Bonferroni multiple comparisons tests, to distinguish statistically significant differences in reporter response between test conditions, are too numerous to be included in the figure panels, but those of importance are mentioned in the text

of the three most toxic divalent metals present, though much of the response is attributable to the high concentration of zinc (Fig. 3a). A somewhat different pattern of response is evinced by *sod-4*; here, both 8 and 5 M mixtures are very similar to P79 at all time points ( $p > 0.05$ ), whereas the responses to 3 M ( $p < 0.01$  at all time points) and Zn ( $p < 0.05$  at 16 and 28 h, but  $p > 0.05$  at 4 h) are markedly lower (Fig. 3b). Thus for *sod-4*, the presence of trivalent metals seems to augment the effect of divalent metals such as zinc. Both *hsp-16.2* and *mtl-2* (Fig. 3c and d) show broadly similar patterns of response to *cyp-35A2* (Fig. 3a), with 3 M and particularly Zn responses being lower than P79 at the early time point but higher at intermediate and/or late time points. For *hsp-16.2*, higher variance for the 3 M response makes these comparisons non-

significant ( $p > 0.05$ ) at 28 h, whereas Zn gives significantly higher ( $p < 0.01$ ) responses in comparison to 5, 8 M and P79 at this time point (Fig. 3c). For *mtl-2*, responses to P79 do not differ significantly from those to 5 or 8 M at either 16 or 28 h ( $p > 0.05$ ), nor indeed to Zn at 16 h ( $p > 0.05$ ; Fig. 3d). However, the response to 3 M is significantly greater than that of 8 M at 16 h ( $p < 0.01$ ), and this applies to both 3 M and Zn in comparison to 5 and 8 M at 28 h ( $p < 0.05$ ).

Table 4 briefly summarises the expression ratios obtained for the same four transgenes (*cyp-35A2*, *sod-4*, *hsp-16.2* and *mtl-2*) using two undiluted samples of control soil pore water—from AR and WL topsoils respectively, alongside repeat tests on tenfold diluted samples of P79 soil pore-water as a positive control. In no case does the apparent

**Table 4** Response of four transgenic strains to control (AR and WL) and P79 soil pore-water samples

Gene	Time							
	Intermediate (16 h) Soil				Late (30 h) Soil			
	UW	AR	WL	P79 (tenfold diluted)	UW	AR	WL	P79 (tenfold diluted)
cyp-35A2	1.00 ± 0.04	1.13 ± 0.04 <i>p</i> > 0.05	1.00 ± 0.09 <i>p</i> > 0.05	<b>2.47 ± 0.04</b> <i>p</i> < 0.01	1.00 ± 0.05	1.10 ± 0.02 <i>p</i> > 0.05	0.97 ± 0.09 <i>p</i> > 0.05	<b>2.97 ± 0.06</b> <i>p</i> < 0.01
sod-4	1.00 ± 0.01	<b>1.16 ± 0.04</b> <i>p</i> < 0.01	<b>1.15 ± 0.03</b> <i>p</i> < 0.01	<b>2.92 ± 0.04</b> <i>p</i> < 0.01	1.0 ± 0.03	0.99 ± 0.03 <i>p</i> > 0.05	1.00 ± 0.01 <i>p</i> > 0.05	<b>3.13 ± 0.05</b> <i>p</i> < 0.01
hsp-16.2	1.00 ± 0.04	<b>1.20 ± 0.03</b> <i>p</i> < 0.01	<b>1.21 ± 0.05</b> <i>p</i> < 0.01	<b>2.58 ± 0.06</b> <i>p</i> < 0.01	1.00 ± 0.03	<b>1.23 ± 0.01</b> <i>p</i> < 0.01	1.10 ± 0.03 <i>p</i> > 0.05	<b>2.98 ± 0.07</b> <i>p</i> < 0.01
mtl-2	1.00 ± 0.02	<b>1.22 ± 0.03</b> <i>p</i> < 0.01	1.09 ± 0.04 <i>p</i> > 0.05	<b>2.55 ± 0.08</b> <i>p</i> < 0.01	1.00 ± 0.02	<b>1.24 ± 0.01</b> <i>p</i> < 0.01	<b>1.13 ± 0.05</b> <i>p</i> < 0.05	<b>2.58 ± 0.07</b> <i>p</i> < 0.01

All data are normalised as expression ratios relative to the corresponding UW control (=1.00). P79 soil pore-water was tested at a 1 in 10 dilution using the same 4 strains as Fig. 3, whereas AR and WL soil pore water samples were undiluted. All *p* values are from Dunnett's multiple comparisons tests (relative to UW controls), and statistically significant expression changes are shown in bold

induction by AR or WL pore-water exceed 25% above the UW controls, although tight error bars mean that some of these small changes in reporter gene expression do reach statistical significance (though not our normal criterion of a 50% expression change). But in all cases these expression changes are far lower than those seen with the tenfold dilution of P79 soil pore-water used as a positive control (2.5–3.1 fold). This confirms that relatively high concentrations of dissolved metals are required to induce stress-reporter expression in our transgenic worms, consistent with our dose–response data for single metals (Fig. 1; Tables 1 and 2; Supplementary Material).

As a further control for this study, we checked whether any of these metals might simply interfere with gene expression generally, or with GFP folding and fluorescence more specifically. To a large extent, this is implicit in the expression patterns documented here, since genes that are strongly induced by one metal often show little or no response to others, and GFP is used as a reporter throughout our study. To reinforce this conclusion, we utilised PD4251 control worms, which constitutively express a *myo-3* muscle-myosin promoter driving abundant GFP reporter expression in the mitochondria of muscle cells throughout the body. As shown in Table 5, for the most part there is little change in *myo-3::GFP* expression at high concentrations of all metals tested, whereas parallel positive controls (using a strongly responsive transgene for each metal) show generally similar expression changes to those seen previously (Table 2 and Supplementary Material). In no case does the change in *myo-3::GFP* expression in PD4251 worms exceed 30% relative to UW controls, though on occasion these changes do attain statistical significance. By contrast, the stress-responsive transgenes tested in parallel all show significantly increased expression (up-regulation by ~1.4–3.5 fold), except in the case

of  $\text{Mn}^{2+}$ ; note, however, that the responses to  $\text{Fe}^{3+}$  and  $\text{Ni}^{2+}$  fall below our usual 50% threshold, due in part to the lower test concentration of  $\text{Fe}^{3+}$  used here to avoid extensive lethality among the PD4251 worms. Thus the changes in stress-responsive transgene expression documented here are specific for each metal and transgene tested, and do not reflect altered expression of genes that are constitutively active.

## Discussion

This paper presents a broad overview of the patterns of stress-response evoked by metal ions in the model nematode, *C. elegans*. One major advantage of using multiple transgenic GFP-reporter strains is that they facilitate high-throughput, low-cost screening of toxicant effects across multiple stress pathways. Although this paper focuses on metal effects, the same approach can be used on pesticides and other environmental toxicants, including nanomaterials (Blinova et al. 2010; Ma et al. 2009b). Far greater detail would be obtainable using gene-arrays to investigate global or pathway-specific patterns of gene expression (transcriptome analysis), but this would be prohibitively expensive for testing many different toxicants at multiple doses and time points, let alone extending such work to mixtures. GFP-reporter responses can be used for initial screening to identify unusual or interesting patterns of response (whether to single toxicants or mixtures), which could then be further investigated using gene arrays (de Pomerai et al. 2008). The responses described here are robust, reproducible and easily measured in a microplate format, lending themselves readily to high-throughput screening methods. It might seem advantageous to omit transfers between 24 and 96-well plates, by conducting

**Table 5** Responses of a constitutively expressed reporter gene (*myo-3::GFP*) to 11 test metals

Metal	Time			
	Intermediate (8–16 h)		Late (20–30 h)	
Strain	<i>myo-3::GFP</i> control	<i>hsp-16.1::GFP</i> SR1	<i>myo-3::GFP</i> control	<i>hsp-16.1::GFP</i> SR1
Cd <sup>2+</sup>	0.99	<b>1.36</b>	0.98	<b>1.76</b>
22 mg l <sup>-1</sup>	<i>p</i> = 0.76	<i>p</i> < <b>0.0001</b>	<i>p</i> = 0.46	<i>p</i> < <b>0.0001</b>
Ni <sup>2+</sup>	1.00	1.05	1.08	<b>1.42</b>
200 mg l <sup>-1</sup>	<i>p</i> = 0.93	<i>p</i> = 0.50	<i>p</i> = 0.17	<i>p</i> = <b>0.009</b>
Strain	<i>myo-3::GFP</i> control	<i>hsp-16.2::GFP</i> SR2	<i>myo-3::GFP</i> control	<i>hsp-16.2::GFP</i> SR2
Fe <sup>3+</sup>	0.90	<b>1.34</b>	<b>0.74</b>	1.04
25 mg l <sup>-1*</sup>	<i>p</i> = 0.07	<i>p</i> < <b>0.0001</b>	<i>p</i> = <b>0.002</b>	<i>p</i> = 0.68
Cu <sup>2+</sup>	<b>0.93</b>	<b>1.63</b>	<b>0.89</b>	<b>2.19</b>
15 mg l <sup>-1</sup>	<i>p</i> = <b>0.007</b>	<i>p</i> = <b>0.003</b>	<i>p</i> = <b>0.002</b>	<i>p</i> < <b>0.0001</b>
As <sup>3+</sup>	0.87	<b>1.75</b>	ND	ND
50 mg l <sup>-1*</sup>	<i>p</i> = 0.12	<i>p</i> < <b>0.0001</b>		
Strain	<i>myo-3::GFP</i> control	<i>mtl-2::GFP</i> SR3	<i>myo-3::GFP</i> control	<i>mtl-2::GFP</i> SR3
Hg <sup>2+</sup>	0.99	<b>2.36</b>	<b>0.86</b>	<b>2.07</b>
10 mg l <sup>-1</sup>	<i>p</i> = 0.90	<i>p</i> < <b>0.0001</b>	<i>p</i> = <b>0.05</b>	<i>p</i> < <b>0.0001</b>
Cr <sup>6+</sup>	<b>0.82</b>	<b>1.42</b>	<b>0.73</b>	<b>1.42</b>
40 mg l <sup>-1</sup>	<i>p</i> = <b>0.004</b>	<i>p</i> = <b>0.0003</b>	<i>p</i> < <b>0.0001</b>	<i>p</i> = <b>0.0002</b>
Strain	<i>myo-3::GFP</i> control	<i>gst-1::GFP</i> SR4	<i>myo-3::GFP</i> control	<i>gst-1::GFP</i> SR4
Zn <sup>2+</sup>	0.95	<b>2.99</b>	0.96	<b>3.46</b>
200 mg l <sup>-1</sup>	<i>p</i> = 0.29	<i>p</i> < <b>0.0001</b>	<i>p</i> = 0.41	<i>p</i> < <b>0.0001</b>
Al <sup>3+</sup>	0.98	<b>2.85</b>	<b>0.82</b>	<b>2.79</b>
50 mg l <sup>-1*</sup>	<i>p</i> = 0.41	<i>p</i> < <b>0.0001</b>	<i>p</i> = <b>0.0009</b>	<i>p</i> < <b>0.0001</b>
Strain	<i>myo-3::GFP</i> control	<i>cyp-34A9::GFP</i> SR5	<i>myo-3::GFP</i> control	<i>cyp-34A9::GFP</i> SR5
Pb <sup>2+</sup>	<b>1.11</b>	<b>1.60</b>	0.94	<b>1.58</b>
50 mg l <sup>-1</sup>	<i>p</i> = <b>0.003</b>	<i>p</i> = <b>0.0002</b>	<i>p</i> = 0.38	<i>p</i> < <b>0.0001</b>
Strain	<i>myo-3::GFP</i> control	<i>hsp-60::GFP</i> SR6	<i>myo-3::GFP</i> control	<i>hsp-60::GFP</i> SR6
Mn <sup>2+</sup>	0.93	<b>1.17</b>	1.15	<b>1.19</b>
500 mg l <sup>-1*</sup>	<i>p</i> = 0.60	<i>p</i> = <b>0.004</b>	<i>p</i> = 0.15	<i>p</i> = <b>0.002</b>

PD4251 control worms (constitutively expressing *myo-3::GFP*) were tested alongside selected stress-responsive transgenic strains (SR1–6) at high doses of all 11 test metals. SR1, used for Cd and Ni, was *hsp-16.1::GFP*; SR2 (Fe, Cu and As) was *hsp-16.2::GFP*; SR3 (Hg and Cr) was *mtl-2::GFP*; SR4 (Zn and Al) was *gst-1::GFP*; SR5 (Pb only) was *cyp-34A9::GFP*; and SR6 (Mn only) was *hsp-60::GFP*. Strain PD4251 proved much more sensitive to Fe, and to some extent also to As and Al, as compared with wild-type N2 or any of the test transgenic strains, and the maximum concentrations tested were therefore reduced by 50% (As and Al) or by 75% (Fe). In the case of Mn, where minimal effects were noted at 200 mg l<sup>-1</sup> (Table 2), the test dose was increased to 500 mg l<sup>-1</sup>. These altered test concentrations are indicated by an asterisk against the metal concentration. Each test condition was normalised against its own UW control as 1.00 (data not shown) when calculating the expression ratios tabulated here. Statistically significant changes in expression ratio (*p* < 0.05; Student's *t* test) are highlighted in bold. Results are shown for intermediate (8–16 h) and late (20–30 h) time-points only, since few changes were seen at early (4–6 h) time points

ND not determined

both exposures and fluorescence measurements in the latter format. In practice, however, the depth of medium in each well often leads to anoxia after 8–20 h, causing expression levels to rise even in UW controls. The patterns of stress-reporter induction reported here are specific for each metal (Table 2), and there is no consistent effect of these metals on constitutive *myo-3::GFP* expression (Table 5).

There are also some minor drawbacks to the reporter approach described here. Extensive coverage of the heat-shock, metal-response and oxidative stress pathways is

feasible, since these gene families are all fairly small, but this breaks down for the much larger gene families involved in xenobiotic metabolism, with >80 phase I *cyp* genes and >40 phase II *gst* genes in *C. elegans*; the 3 *cyp* and 2 *gst* genes tested here are all known to be stress-inducible, but represent only the tip of a large iceberg. Also excluded are toxicant-specific responses, such as the *cdr* genes induced by cadmium exposure (Dong et al. 2005) or the *aip-1* gene induced by arsenite (Sok et al. 2001). Our approach uses transcriptional fusions and a stable GFP



variant, and thus can only reliably measure the induction of stress-response gene expression; subtle expression changes involving internal or 3' regulatory sequences and mRNA or protein stability will be missed. Because GFP needs to be translated, folded and auto-oxidised before it becomes fluorescent, there will be a time lag ( $\geq 1$  h) before the underlying transcriptional responses become apparent as increased fluorescence, a problem compounded by background signal from auto-fluorescent granules in the gut (David et al. 2003). Finally, using mixed-stage cultures might miss important developmental changes in the patterns of stress-gene expression, though this could be readily addressed by using synchronised cultures.

The data presented here show that high doses of most metals tested induce multiple stress responses (Table 2 and Supplementary Material). As expected, toxic metals induce heat-shock proteins (reflecting proteotoxic stress), oxidative stress enzymes (reflecting increased levels of reactive oxygen species, ROS) and mtl (involved in sequestering heavy metals). Unexpected, but not unprecedented (e.g. Cui et al. 2007), was the observation that most metals also induce one or more *cyp* P450s involved in phase I xenobiotic metabolism—even though these enzymes normally modify organic substrates (e.g. by epoxidation of aromatic ring structures) and presumably cannot deal with metals directly. This may reflect indirect metal inhibition of key metabolic pathways, or possibly transcriptional co-regulation of certain *cyp* genes (e.g. *cyp-34A9*) along with other stress-response genes (*mtl-1*, *sod-3* and *hsp-16.1/16.2*) by master regulators such as DAF-16 (Murphy et al. 2003) or SLR-2 (Kirienko and Fay 2010). The functional role (if any) of *cyp* gene expression under conditions of metal stress remains obscure.

One consistent observation (Table 2 and Supplementary Material) is that the genes encoding key TFs are in most cases far less strongly induced by metal exposures than the output genes whose transcription they control. This applies to *daf-16*, *skn-1*, *elt-2* and possibly *cep-1*, but is most obvious in the case of *hsf-1*, whose expression level varies far less than that of its downstream targets (such as the heat-inducible *hsp-70* and *hsp-16.1/16.2* genes). Most if not all of these TFs are expressed constitutively in *C. elegans*, and they exist predominantly in an inactive form in unstressed cells (e.g. hyperphosphorylated DAF-16). Stress conditions result in their post-translational modification (dephosphorylation of DAF-16) and activation, with subsequent binding to DNA response elements near the downstream target genes. Thus the primary up-regulation of these target genes is not dependent upon prior up-regulation of the requisite TF gene(s). It is possible that such up-regulation occurs occasionally as a last-ditch defensive strategy (to maximise expression of the downstream targets), but this is not supported by an examination

of Table 2, where up-regulation of any particular TF gene does not always correlate with very strong induction of its target genes.

The predictions of our mathematical model of the *mtl* gene circuit (Haque 2011) in relation to simple two-component mixtures of similar versus dissimilar metal ions have been largely confirmed by experiment (Fig. 2). Broadly speaking, our *mtl* model envisages that complex formation between divalent metal inducers and both *U* and *MTL* proteins should be rapid, leading to additive effects (Fig. 2a)—whereas dissimilar ions (where one is unable to bind efficiently to either or both proteins) are expected to slow down one or both rates of complex formation, leading to interference (Fig. 2b). Although our RNAi evidence implicating ELT-2 as a key transcriptional regulator of this gene circuit is currently stronger for *mtl-1* than for *mtl-2* (unpublished data), the predictions of our model would not change if another TF were involved in regulating *mtl-2*, so long as the patterns of metal:protein interaction and complex formation are broadly the same as that proposed by Moilanen et al. (1999) for ELT-2. We have only tested this model in relation to the output (*mtl*) genes, so formally any TF with similar properties could replace ELT-2 in the model. The experimental tests shown in Fig. 2c and d confirm our model predictions for *mtl-2* for both additive (Hg + Cd) and interfering (Hg + Fe) effects, and furthermore confirm the dynamic predictions as to how these additive or interfering effects should evolve over time. These patterns of response to simple mixtures seem in turn to bear some relationship to those seen with more complex mixtures.

Looking at the soil pore water samples and metal mixture tests, it is clear that artificial mixtures of metals closely mimic the effects of the diluted P79 soil–water samples derived from a mine site where metals are the major soil contaminants. There are interesting fluctuations in the strength of gene induction as this mixture is simplified from eight metals down to one (Zn). There is some evidence for additive toxicity in the case of *sod-4* (encoding an extracellular superoxide dismutase), but interference effects can be discerned for the other genes tested in Fig. 2 (*cyp-35A2*, *mtl-2* and *hsp-16.2*). In Fig. 3, the effects of Zn and 3 M (divalent metals only) exceed those of the 5 M (containing  $\text{Al}^{3+}$ ) or 8 M (also containing  $\text{Fe}^{3+}$ ) mixtures for one or more time points for 3 out of the 4 genes tested. Furthermore, in most cases, the effects of 3 M are significantly ( $p < 0.05$ ) greater than those of Zn alone, suggesting additive effects for the other two divalent ions (Cu and Cd) included in 3 M. For *mtl-2*, there is a significantly ( $p < 0.05$ ) greater effect of 3 M as compared to Zn alone at 16 h, consistent with additive effects, although this is not seen at 28 h. There are also some suggestions that interference between dissimilar metals in the more complex

mixtures (5 and 8 M) may have moderating effects on reporter-gene expression. Looking specifically at *mtl-2*, significantly ( $p < 0.05$ ) smaller effects are seen for both 5 M (+Al<sup>3+</sup>) and 8 M (+Fe<sup>3+</sup>) mixtures, as compared to 3 M (16 and 28 h) and Zn (28 h only), consistent with the mathematical model predictions for simple 2-component mixtures shown in Fig. 2. This suggests that modelling predictions based on simple mixtures might also be applicable to more complex mixtures.

Could similar mechanisms be invoked to explain the rather similar patterns of response seen for *cyp-35A2* and *hsp-16.2*? Heat-shock proteins are regulated by a feedback loop that is formally similar to our model for regulation of the *mtl* genes, although it is unclear why trivalent metal ions should differ in any way from divalent ions in terms of the proteotoxic damage they inflict. As for *cyp* genes, their mechanism of induction remains obscure in *C. elegans*, despite the possible involvement of the NRH-8 orphan nuclear receptor (Lindblom et al. 2001). An apparent outlier in terms of response is *sod-4*, which may be regulated by SKN-1 as part of the wider oxidative stress response (An and Blackwell 2003). However, we have not so far modelled this regulatory gene circuit, and *sod-4* (in contrast to *sod-1*) is not among the SKN-1-dependent genes that are strongly up-regulated by oxidative stress (Park et al. 2009), although it does possess SKN-1 binding sites (Back et al. 2010).

We have measured the concentrations of several other metals (Na, Mg, K, Ca) which might potentially enhance or inhibit the effects of these metal mixtures. Na and K concentrations are well within the salinity tolerance range for *C. elegans* (up to 15 g l<sup>-1</sup> for Na and 11 g l<sup>-1</sup> for K; Khanna et al. 1997). Calcium (and possibly magnesium) can inhibit the uptake of heavy metals into *C. elegans* (Güven et al. 1995), yet P79 soil pore water is still extremely toxic. It remains possible that competition between different ions might limit metal entry into cells—although the effects of 3 M (usually higher than Zn alone) argue against this, and there is little if any net effect of the additional metals present in 8 M or indeed P79 as compared to the 5 M mixture. Furthermore, the very different response pattern seen for *sod-4* (whose expression broadly increases in line with the number of metals present, at least up to 5 M) cannot be explained in these terms. A direct approach to this question would involve measuring metal levels in exposed worms; however, metal adsorption to the cuticle necessitates at least a crude fractionation of the worms, so complicating the analysis (Power and de Pomerai 1999).

Overall, this study shows that using multiple GFP stress-reporter strains of *C. elegans* can provide an overview or outline map of the patterns of gene response to toxic environmental chemicals, both singly and in mixtures.

Such maps are inevitably sketchy in comparison to the insights obtainable from gene arrays, but the GFP assay methodology is far simpler and cheaper, and the outcomes are usually amenable to more straightforward interpretation, since the functions of most of these response genes are already well characterised. Furthermore, data consistency from the GFP assays allows statistical significance to be inferred for gene-expression changes of the order of 50% or less, where gene arrays are notoriously subject to noise and false positives or negatives. The GFP reporter approach can easily be extended to further stress-response genes, to drugs, pesticides or other organic pollutants, and to field samples as well as laboratory mixtures or single chemicals. It is our contention that mixture effects on gene expression (though not necessarily on overall toxicity) should in principle be predictable on the basis of mathematical models describing the underlying gene circuits (de Pomerai et al. 2008) and how they respond to component single toxicants. One surprising outcome of our *mtl* model (Fig. 2) was the prediction of interfering effects for chemically dissimilar metal ions as well as the expected additive effects for similar metals. It remains to be seen whether such interference effects in metal mixtures are widespread, whether they occur also with other toxicants (such as pesticides), and whether they can also influence general toxicity (in terms of mortality, or whole-organism endpoints such as brood size, motility or feeding inhibition) as well as stress-gene expression.

**Acknowledgments** The authors are grateful to the British Council's UK-IERI scheme (Major Award MA-05) for financial support to the Nottingham researchers, and Project CGL2009-14686-C02 in support of the ECOMETRISK project (Spanish research team). David Baillie holds a Canada Research Chair and is supported by a Discovery grant from NSERC Canada. Dr Liz Bailey (Environmental Science Division, School of Biosciences, University of Nottingham) is thanked for the ICP-MS analyses. Declan Brady is thanked for expert technical assistance.

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